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TITLE: Macrophage Efferocytosis and Prostate Cancer Bone Metastasis

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14. ABSTRACT

Macrophages play a vital role in maintaining tissue homeostasis by clearing apoptotic cells through a specialized form of phagocytosis termed efferocytosis. The translation of this functional role during pathophysiological states such as tumor metastasis to the skeleton is unknown. The purpose of this project was to determine the efferocytic myeloid cell type, its role in supporting prostate cancer (PCa) skeletal metastasis, and its potential for targeting for therapeutic purposes. Molecular mechanisms of macrophage efferocytosis were analyzed using murine bone marrow macrophages where $F4/80^{+/}CD206^{+}$ positive cells (M2 macrophages) were found proficient at efferocytosis of apoptotic tumor cells and PS coated apoptotic mimicry beads. M2 polarization increased macrophage efferocytosis, and correlated with increased pro-tumorigenic, M2-like gene expression (YM1, TGF-β). Soluble factors released from macrophages during efferocytosis resulted in a 30% increase of PC-3 cell numbers, compared to non-efferocytotic macrophages. Using two different prostate cancer mouse models, treatment with trabectedin, resulted in diminished M2 like macrophages that were $TRAILR2^{+}$ and $CD115^{+}$ and decreased skeletal metastasis. Clinical analysis of peripheral blood mononuclear cells isolated from patients with PCa skeletal metastasis showed a 20% increase in triple positive CD68+/CD14+/CD16+ (alternatively activated, M2) monocyte populations as compared with non-cancer controls which significantly correlated with their Gleason score. Moreover, monocytes isolated from PCa skeletal metastasis patients presented a 2.5 fold increase in efferocytosis versus patients without cancer. To further confirm these findings, we analyzed tissue microarrays, which revealed high risk (≥8 Gleason) PCa tissue expressed significantly higher levels of CD68, in invasive versus benign tissues. These findings suggest M2 like monocytes and macrophages promote prostate cancer skeletal metastasis by active engagement in efferocytosis.

15. SUBJECT TERMS

None Provided

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INTRODUCTION: Despite the advancement of prostate cancer (PCa) research, little is known about the functional role of tumor-associated macrophages in promoting tumor growth in the bone microenvironment. A subpopulation of alternatively activated macrophages, is associated with supporting tumor growth, but the interaction between macrophages and tumor cells in the context of the bone microenvironment is unexplored. Macrophages play a vital role in maintaining tissue homeostasis, by clearing foreign pathogens and apoptotic tumor cells through phagocytosis. As a result of apoptotic cell phagocytosis (a specialized process termed **efferocytosis**), macrophages release transforming growth factor beta, to prevent a pro-inflammatory response. Interestingly, cancer cells exploit this normal process in order to evade the immune system and substantiate its growth. However, the role of bone resident macrophages (osteal macrophages), in supporting tumor growth via efferocytosis has not been explored.

Hypothesis: The hypothesis of this proposal is that osteal macrophages/monocytes support prostate cancer bone metastasis through the phagocytosis of apoptotic tumor cells (efferocytosis).

Specific Aims:

1. To identify the phagocytic/efferocytic macrophage population in the tumor microenvironment of prostate bone metastases and determine its ability to support tumor growth.
2. To determine the impact of macrophage derived transforming growth factor beta, secreted upon efferocytosis, on prostate cancer bone metastasis.

1. **KEYWORDS:** skeletal metastasis, macrophages, monocytes, trabectedin, efferocytosis

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Task 1: Training and educational development in prostate cancer research

1a. Present ongoing research at monthly prostate cancer project group (PO1) meeting (months 0-4)

This task was accomplished. Meetings at the monthly PO1 group were very beneficial in helping the project to progress forward.

1b. Attend a manuscript writing workshop series at the University of Michigan entitled "Preparing Manuscripts for Publication" (months 0-4)

This manuscript-writing workshop was very beneficial and offered many tips in my writing as an independent researcher. This task was completed in full.

1c. Attend national scientific meetings such as ASBMR and AACR, which are relevant to prostate cancer research. (months 0-10)

The results from this project rendered three different opportunities to attend National/International conferences to present the project to a diverse audience. The conferences resulted in travel awards with oral and poster presentations.

(1) ASBMR 2014. Plenary Speaker Session (Houston TX)
(2) ASIP EB 2015. Oral and Poster Presentation (Boston, MA)
(3) Case Western University AUMF Symposium- Oral Presentation (Cleveland, OH)

1d. Participate as a guest lecturer in relevant courses at the University of Michigan (months 0-4)
During year 1, I participated as a guest lecturer on osteoclasts with Dr. Laurie McCauley.

Task 2: To identify the phagocytic/efferocytic macrophage population in the tumor microenvironment of prostate bone metastases and determine its ability to support tumor growth. (Months 0-4)

2a: Identify the efferocytic MΦ population in the bone marrow**.**

Timeline: months 1-4

This task is completed and a manuscript is in the final stages of preparation for submission to Cancer Research.

Using flow cytometry, we identified a unique macrophage population in mouse bone marrow that was F4/80$^{+/}$CD206^{+}, M2-like macrophages (Figure 1). This subset of macrophages was more proficient at efferocytosis of apoptotic tumor cells and phosphatidylserine coated apoptotic mimicry beads than the classical M1-like macrophage population (F4/80^{+}/CD86^{+}), prevalent in the bone marrow.

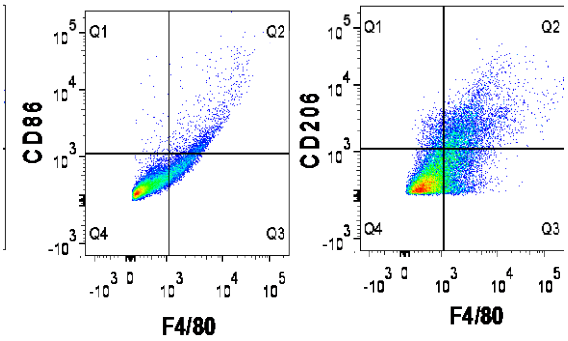

Figure 1

Figure 1. Cell surface markers F4/80, CD86 and CD206 were used to identify polarized macrophage populations using flow cytometry. Data shown is representative FACs images.

2b: Investigate MΦ efferocytosis in prostate cancer bone metastasis

Timeline: months 0-4

This task is completed and results are the manuscript soon to be submitted to Cancer Research.

Briefly, bone marrow macrophages were primed with tumors and targeted using the pharmacologic compound trabectedin, a therapeutic agent that targets tumor-associated macrophages. An orthotropic bone model for prostate cancer in mice was used to study the effects of trabectedin. A single administration of trabectedin 7 days prior to tumor inoculation resulted in decreased tumor bioluminescence in tibiae as early as day 21 (Figure 2A). Mice bearing tumors and treated with trabectedin for 6 weeks showed a significant decrease in F4/80^{+},

CD206^{+} and F4/80^{+}/CD206^{+} double positive cells (Figure 2B). Similar results were also found using an experimental skeletal metastasis model.

Figure 2

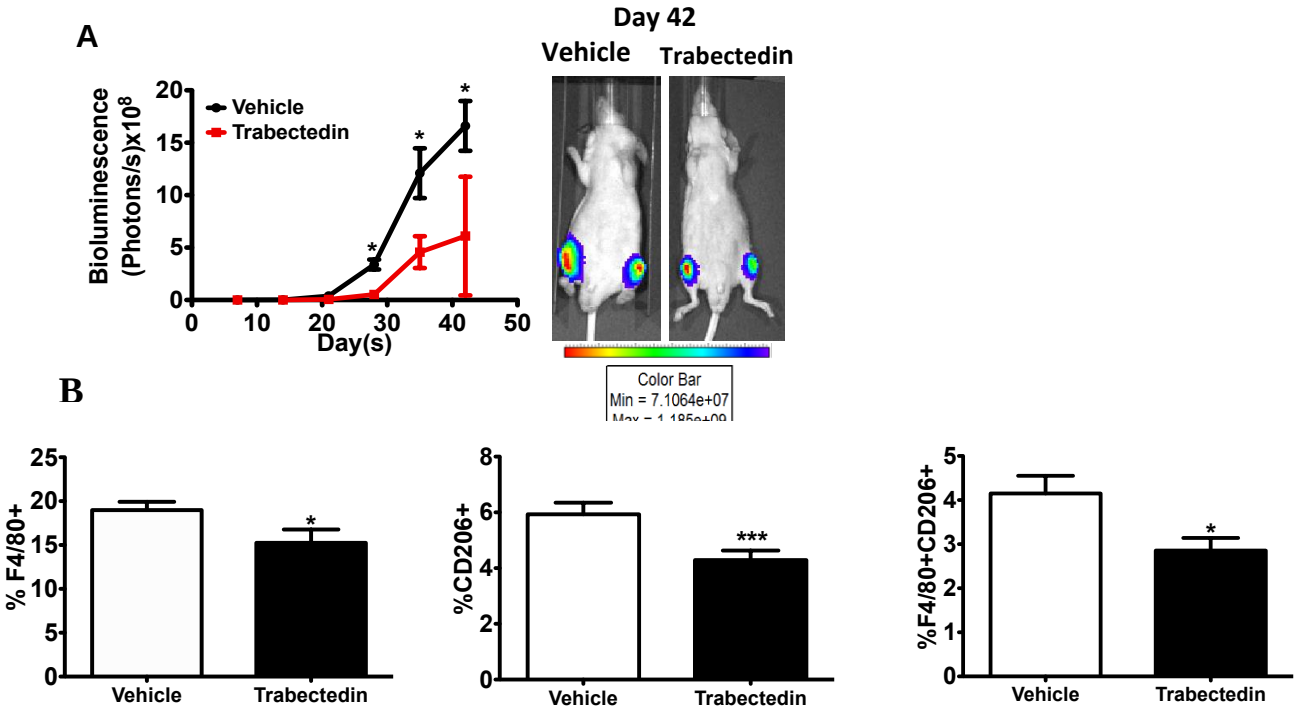

Figure 2. Ablation of M2 like bone marrow cells retards prostate cancer tumor growth. A. Male athymic mice were divided into two groups and treated with a single injection of saline control (n=10) or trabectedin (n=8). Seven days after initial treatment (0.15 kg/mg/bodyweight), PC-3^{Luc} cells were injected into the bone marrow space of the left and right tibiae, and mice followed for 42d. Tumor growth in the hind limbs was measured weekly using bioluminescence. **B**) Bone marrow cells were isolated and analyzed for markers F4/80, CD68, CD86, and CD206 using flow cytometric analysis (with representative flow cytometric analyses). Data are mean ± S.E., *p<0.05.

2c: Monocyte profiling in human prostate cancer patients

Timeline: months 0-4

This task has been completed and included in our manuscript in preparation for Cancer Research. We obtained an array of prostate cancer tissue including bone metastasis (N=72) and stained the tissue for the phagocytic macrophage marker, CD68 (Figure 3A). The stained tissue was analyzed for CD68 expression. There was a significant increase (p<0.001) in CD68 positive cells

in more invasive tissue, especially in high-grade tumors (Figure 3B). We also investigated the presence of a unique monocyte population, M2 monocytes ($CD68^+/CD14^+CD16^+$), in the peripheral blood of prostate cancer skeletal metastasis patients versus healthy, non-cancerous patients. Interestingly, significantly higher levels of M2 monocytes were identified in patients with skeletal metastasis ($p<0.001$) compared to the normal controls (Figure 3C). Freshly isolated human monocytes from skeletal metastasis patients were more adept at apoptotic cell efferocytosis than non-cancerous patients. Moreover, this monocyte population also correlated with a high Gleason score. Our data indicate a unique correlation between M2-like phagocytic monocytes in advanced prostate cancer patients.

Figure 3

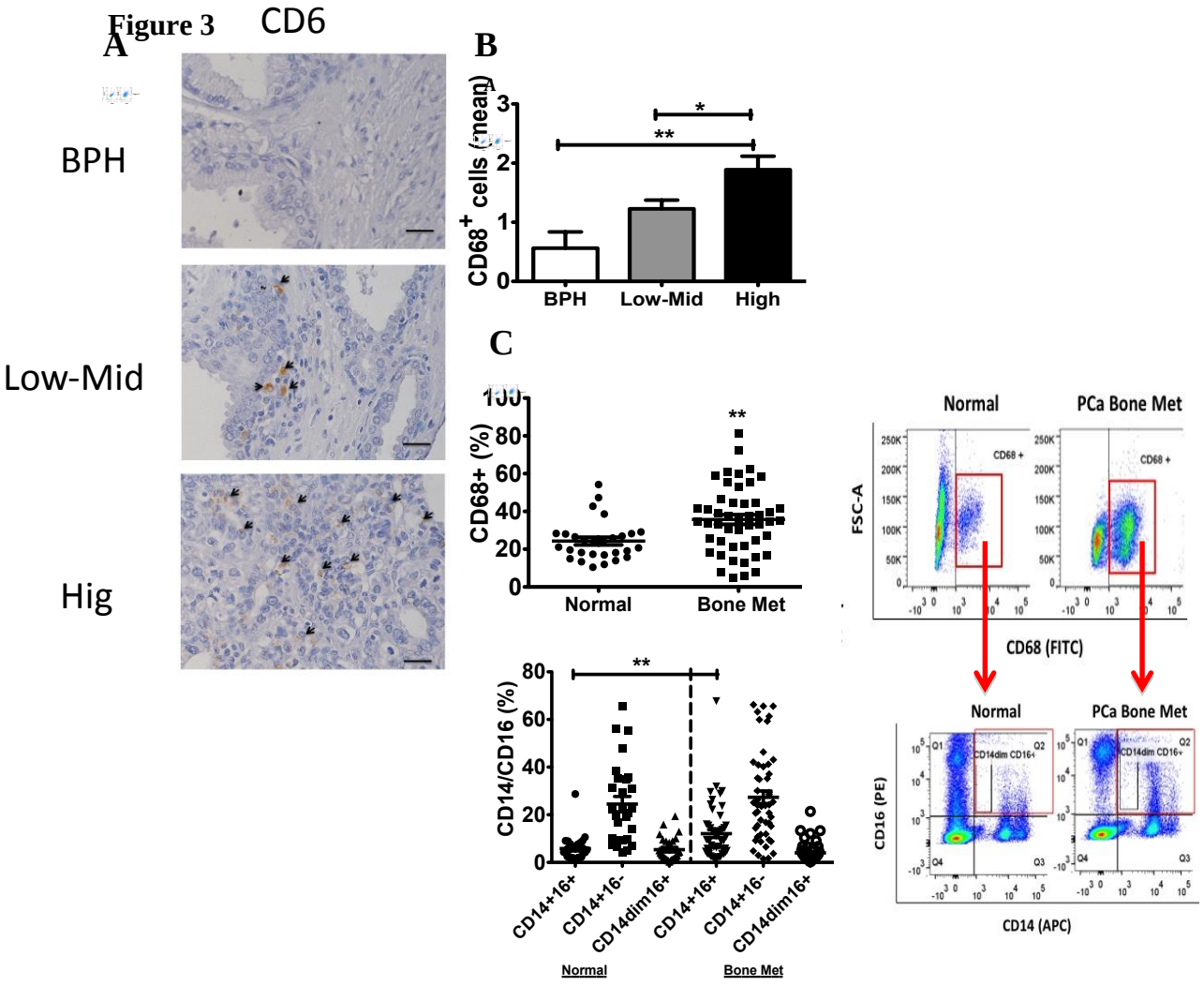

Figure 3. M2-like peripheral blood mononuclear populations are associated with human prostate cancer skeletal metastasis. A. Representative images of immunohistochemistry of prostate cancer tissue microarray specimens including BPH (n=16), low to mid risk (n=22) (≤7 Gleason) and high risk (n=36) (≥8 Gleason) for $CD68^+$ cells. Images are taken at 400-fold magnification. **B.** Quantitative analysis of tissue specimens for the sum of CD68+ cells in four different fields of view. Representative images were taken at 20X for analysis. Data are mean ± S.E., *p<0.05, **p<0.01. **C.** Monocytes were isolated from whole peripheral blood of non-cancer (n=24) and prostate cancer bone metastatic patients (n=50). Monocyte populations were assessed via flow cytomteric analysis. CD68+ monocytes were assessed and representative FACS images displayed at right. Data are mean ± S.E., **p<0.01 vs. normal controls. Sub-populations $CD14^+CD16^+$, $CD14^+CD16^-$ and $CD14^{dim}CD16^+$ were gated off of $CD68^+$ population for triple positive cells. Representative images are shown above. Data are mean ± S.E., **p<0.01 vs. normal controls.

Task 3: To determine the impact of macrophage derived TGFβ, secreted upon efferocytosis, on prostate cancer bone metastasis. (Months 13-24)

This task has not been completed yet. However, we have made progress in the successful breeding of the **TGFβ Cre/Lox** mice and experiments using these mice can be completed in the near future. Completion of this task will render further insight into the role of TGF**β in macrophage efferocytosis in prostate skeletal metastasis.**

How were the results disseminated to communities of interest?
The data received from this grant have been presented at two national meetings and will soon be submitted to Cancer Research.

What do you plan to do during the next reporting period to accomplish the goals?

Pending the identification and approval of a new post-doctoral fellow, the TGFβ studies using the mouse model will be used to get a more in-depth understanding of the process of efferocytosis in prostate skeletal metastasis.

4. IMPACT: Most men with advanced prostate cancer will develop bone metastatic disease, which can lead to death. Minimal advances have been made to diminish the progression of bone

metastasis. Thus, this is a critical area to explore and develop therapies. While research has focused on the interaction of soluble proteins and genetic alterations, there has been no exploration on the impact of the phagocytic role of macrophages and efferocytosis (phagocytosis of apoptotic tumor cells) in the development of prostate cancer bone metastasis. Studies have shown that as a result of macrophage efferocytosis, they release factors that promote tumor progression and suppress the anti-tumoral immune response, which is needed to prevent tumor growth. Studies in autoimmune diseases, atherosclerosis, and wound healing have identified mediators of efferocytosis as therapeutic targets; however, this has not been evaluated in the context of prostate cancer in bone. Therefore, we identified and characterized the sub-population of monocytes/macrophages that engaged in efferocytosis in prostate cancer bone metastasis. We also identified plausible targets for the inhibition of efferocytic macrophages associated with bone metastases. The results from this study can serve as the foundation for the development and pre-clinical use of targeting the specific macrophage population contributing to tumor growth and how it impacts the bone microenvironment.

What was the impact on the development of the principal discipline(s) of the project?

The current disciplines of the project are prostate cancer and bone immunology. Our current findings from this project identified a unique population of myeloid cells in the bone and peripheral blood (monocytes and macrophages) that engaged in efferocytosis and contributed to PCa tumor cell growth.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Aim 1 Changes/Problems:
We decided to change the drug proposed to target monocytes/macrophages due to the potential difficulties in consistent administration as well as access to the drug. Therefore we worked with a collaborator to use a more clinically relevant drug, trabectedin that has been shown to be effective in sarcoma in human patients in Europe and is currently in clinical trials.

Aim 2 Changes/Problems:
We experienced an initial delay in developing the TGF beta Cre/Lox mouse model for our second aim. While the breeding took longer than our expected time reported in our original proposal, we were able to obtain mice to begin our animal studies for part 2.

6. PRODUCTS:
Abstracts/Conferences:

1. American Society for Investigative Pathology (ASIP) (San Diego, CA) - "Alternatively Activated Monocytes/Macrophages Support Prostate Cancer Skeletal Metastasis"
2. American Society for Bone and Mineral Research (Houston, TX) – "Alternatively Activated Monocyte and Macrophage Efferocytosis Support Prostate Cancer Skeletal Metastasis"
3. Case Western Reserve Research Symposium- "Macrophage Efferocytosis Supports Prostate Cancer Skeletal Metastasis"

Publication(s):

Jones, D.J, Soki, F., Koh, J A., Shiozawa, Y., Pienta, K., Morgan, T., Roca, H., McCauley, L. (2015). Alternatively Activated Myeloid Cells Support Prostate Cancer Skeletal Metastasis via Efferocytsosis Cancer Res. (In prepartion)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	L.C. Hofbauer
Institution:	Department of Endocrinology, Diabetes, and Bone Disease, Dresden University Medical Center, Dresden Germany
Project Role:	Collaborator
Contribution to Project:	Dr. Hofbauer contributed prostate cancer tissue Arrays and Trabectedin (therapeutic drug used in the study)

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The PI, Dr. Jones, has left the University of Michigan and we are currently recruiting another post-doctoral fellow for this project.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS: None

QUAD CHARTS: The Quad Chart (available on https://www.usamraa.army.mil) shall be updated and submitted as an appendix.

9. APPENDICES:

N/A